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Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector

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Summary

Using a recombinant potato virus X (PVX) vector, we investigated the relationship between the length of RNA sequence identity with a transgene and the ability to promote post-transcriptional gene silencing (PTGS) and transgene methylation. The lower size limit required for targeting reporter transgene mRNA *de novo* using PTGS was 23 nucleotides (nt) of complete identity, a size corresponding to that of small RNAs associated with PTGS in plants and RNA interference (RNAi) in animals. The size and sequence specificity were also explored for PTGS-associated transgene methylation and for the targeting of the vector RNA. The PTGS-competent short sequences resulted in similar patterns of methylation. In all cases, including specific sequences of 33 nt with or without symmetrical cytosine residues, the methylation was distributed throughout the transcribed region of the transgene. In contrast, short sequences lacking symmetrical cytosines were less efficient at promoting PTGS of the transgene mRNA. Short *gfp* sequences in the PVX vector provided as effective a target for the degradation of viral RNA as was found for PVX carrying the complete *gfp* cDNA. Short sequences were able to initiate PTGS of an endogenous gene, phytoene desaturase, although this occurred in the absence of DNA methylation. This experimental approach provides important insights into the relationship between short RNA sequences and PTGS.

Keywords: post-transcriptional gene silencing, methylation, transgenes, homology, minimal size, small RNAs.

Introduction

Post-transcriptional gene silencing (PTGS) is based on a homology-dependent degradation of RNA in the cytoplasm. The target RNA may be derived from transgenes, endogenous genes or viruses. Although originally identified in plants as the underlying mechanism obtained from the transgenic expression of virus-derived sequences, PTGS is now recognized as a fundamental process related to a wide range of epigenetic phenomena (reviewed by Depicker and Van Montagu, 1997; Fagard and Vaucheret, 2000; Plasterk and Ketting, 2000; Stam *et al.*, 1997; Van den Boogaart *et al.*, 1998). It is also apparent that PTGS is not restricted to plants, being mechanistically related to quelling in *Neurospora* (Cogoni and Macino, 1999; Cogoni *et al.*, 1996) and RNAi in *Caenorhabditis elegans* (Fire *et al.*, 1998); *Escherichia coli* (Tchurikov *et al.*, 2000); *Drosophila melanogaster* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999); fish (Wargelius *et al.*, 1999; Yx *et al.*, 2000); and mammals (Wianny and Zernicka-Goetz, 2000). Particularly compelling is the involvement of homologous genes in *Neurospora, C. elegans* and *Arabidopsis thaliana* (Cogoni and Macino, 1999; Dalmay *et al.*, 2000a; Mourrain *et al.*, 2000; Smardon *et al.*, 2000) and the association of small RNAs with PTGS and RNAi in plants (Hamilton and Baulcombe, 1999) and *Drosophila* (Hammond *et al.*, 2000; Zamore *et al.*, 2000), respectively. In plants, small RNAs were found associated with silenced transgenes and virus infection; small RNAs of the same size (21 and 23 nt) were shown to activate the homology-dependent degradation of target RNAs in cell free extracts of *Drosophila* embryos, and to generate further similar RNAs as products (Zamore

et al., 2000). For plants, it was also proposed (Hamilton and Baulcombe, 1999) that these small RNAs might constitute cellular signals for the induction of PTGS, both locally and at more distal regions of the plant. However, it has not been shown that such short lengths of RNA are capable of promoting PTGS-mediated targeting of homologous RNA *de novo*.

The potential for RNA to interact with genomic sequences has been shown (Wassenegger *et al.*, 1994), even when the RNA is generated outside the nucleus. Hence infection of transgenic plants with cytoplasmically replicating RNA viruses resulted in *de novo* methylation of the transgene if the viral RNA contained regions of homology with the genomic DNA (Jones *et al.*, 1998, Jones *et al.*, 1999). If the homology corresponded to the transgene promoter, transcriptional gene silencing ensued. Homology corresponding to the transgene mRNA sequence was associated with PTGS. In this case, methylation was restricted to the transcribed region, but spread beyond the initial region of homology (Jones *et al.*, 1999). Although a tight correlation between methylation and PTGS has been shown (English *et al.*, 1996; Ingelbrecht *et al.*, 1994; Jones *et al.*, 1998; Sijen *et al.*, 1996; Van Houdt *et al.*, 1997), the relevance of methylation for PTGS remains uncertain. It has been suggested, however, that methylation could be involved in the amplification and maintenance of transgene-mediated PTGS (Dalmay *et al.*, 2000b; Jones *et al.*, 1999).

In this paper, we have used the ability of potato virus X (PVX) carrying sequences derived from the *Aequoria victoria* green fluorescent protein gene (*gfp*) to silence *gfp* expression in non-silenced *gfp*-transgenic *Nicotiana benthamiana* plants to assess the size and sequence requirements for promoting PTGS of *gfp* mRNA and transgene methylation. The data support the view that short homologous RNA sequences of 23 nt can target PTGS to *gfp* mRNA *de novo*, but that towards the lower size limits efficiency may be influenced by the sequence itself.

Results

Nucleic acid homology of 23 nt is sufficient to direct PTGS to, and de novo *methylation of, a GFP transgene*

To determine the shortest homologous RNA sequence able to target PTGS to *gfp* mRNA, fragments of *gfp* DNA were cloned into a PVX cDNA vector and the virus inoculated to *gfp*-transgenic *N. benthamiana*. The *gfp* fragments, generated by DNaseI digestion, were size selected (<100 bp) before cloning, and the resultant clones were sequenced. The orientation and origin of the fragments are shown in Figure 1(a). Surprisingly, there was a strong 3′ bias in the source distribution of the cloned fragments, although there was an equal distribution of clones in the sense (S) and antisense (AS) orientations (Figure 1a). These cloned fragments were compared with PVX-GFP containing the complete *gfp* cDNA for their ability to direct silencing. The plants were scored visually for silencing of *gfp* expression 25 (Figure 1b), 34 and 41 days post-infection (dpi). Under UV illumination silencing was seen as the loss of green GFP fluorescence to

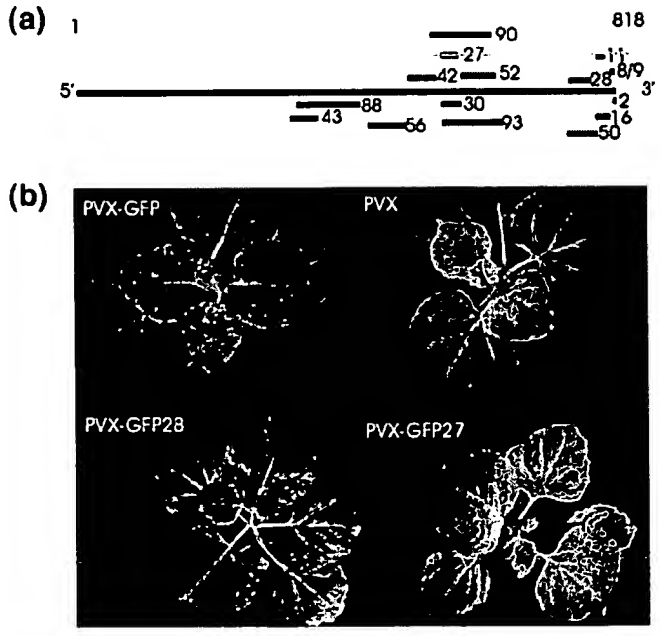

Figure 1. Effect of random *gfp* fragments on directing silencing to *gfp* transgene mRNA.
(a) Origin of random *gfp* DNAse I fragments of <100 nt inserted in the PVX vector for testing as promoters of *gfp* PTGS. Numbers indicate the size of the fragments. Sequences above the line were cloned in the S orientation, those below in the AS orientation.
(b) Phenotypes of plants observed under UV illumination at 25 dpi with PVX-GFP, PVX (no insert), PVX-GFP28 (28 nt) or PVX-GFP27 (27 nt).

Table 1. Silencing of *gfp* with PVX containing 20–30 nt homology to three distinct regions of *gfp* DNA

gfp region	Size (nt)	Nucleotide position	Sense (S/AS)	Silencing (+/–)
Region 1	20	316–335	S	–
	20	316–335	AS	–
	23	316–338	S	–
	23	316–338	AS	+
	27	316–342	S	+
	27	316–342	AS	+
	30	316–345	S	+
	30	316–345	AS	+
Region 2	20	550–569	AS	–
	21	550–570	S	–
	23	550–572	S	–
	27[a]	550–576	S	+
	30[a]	550–579	AS	+
Region 3	20	746–765	AS	–
	22	746–767	S	–
	23	746–768	S	+
	23	746–768	AS	+
	27	746–772	AS	+
	28[a]	746–773	S	+
	30	746–775	S	+
	30	746–775	AS	+

[a]Sequences also tested from the preliminary random fragmentation of *gfp*.

reveal red chlorophyll fluorescence (Figure 1b). The range of fragments required to promote silencing showed a sharp cut-off in size, with fragments of 27 nt and larger being effective, but fragments of 16 nt and less being ineffective. Fragments in either sense or antisense orientation were effective.

Although all fragments of 27 nt and larger were able to direct silencing, there was a marked difference in their relative effectiveness. In contrast to the response to PVX-GFP, which resulted in rapid and complete silencing (full red fluorescence) by 20 dpi, many of the smaller fragments took longer and showed a patchy silencing phenotype in the early stages of the infection. The largest variation was seen at 25 days (Figure 1b) when comparing PVX-GFP27 (27 nt), PVX-GFP28 (28 nt) and PVX-GFP (818 nt). Close to the minimal size for successful silencing, just a 1 nt difference in the length of homologous RNA had a dramatic effect on silencing efficiency. Eventually all the competent RNA fragments produced leaves showing an extensively red fluorescent phenotype.

To obtain a more precise estimate of the size limit for silencing, a targeted approach was taken whereby nested synthetic oligonucleotides of 20, 23 27 and 30 nt to three different regions of *gfp* (nt 316–345, nt 550–579 and nt 746–775) were inserted into the PVX vector. In most cases insertions in both orientations were obtained. With the contribution from the flanking nucleotides from the *Sma*I cloning site, a range of *gfp* homologies of 20–30 nt resulted (Table 1). The recombinant PVX variants were inoculated onto *N. benthamiana* and again scored for the initiation of silencing. After 45 dpi, silencing was seen when the homology was 23 nt or longer (Table 1). There were two exceptions (PVX *gfp* homology 316–338 and 550–572 in the S orientation), which failed to initiate silencing despite having 23 nt *gfp* homology. However, close to the lower limit for silencing, homologous sequences in the AS orientation appeared to be more efficient (data not shown). No viruses with homologous sequences of less than 23 nt initiated *gfp* silencing. Consistent with the random approach, initiation of silencing was slower and patchy with the smaller fragments (data not shown).

Surprisingly, one 27-mer *gfp*-specific oligonucleotide (nt 746–772) did not initiate silencing (data not shown), even though it covered a 23 nt region (nt 746–768) which was competent for silencing (Table 1). However, sequencing of these 27 nt identified an error in the sequence which divided the 27 nt into 12 and 14 nt of identity with *gfp*.

Previously, in the same experimental system (Jones *et al.*, 1999), we had shown that GFP-specific RNA in the PVX vector was able to direct methylation of the transcribed region of the *gfp* transgene, irrespective of whether the 5′ or 3′ regions of the sequence were used as inducers. To see whether the short *gfp* fragments retained their capacity as inducers of methylation, genomic DNA from completely silenced tissues of plants infected with PVX-GFP28 (28S nt), PVX-GFP43 (43AS nt) or PVX-GFP were subjected to analysis using *Sau96*I and Southern blotting with the complete *gfp* cDNA, as before (Jones *et al.*, 1999). *Sau96*I, which has a recognition sequence GGNCC, is sensitive to methylation of canonical or symmetrical cytosine residues (CpG or CpNpG), or non-symmetrical C residues when the nt 3′ to GGNCC is not a G residue. The organization of the 35S:*gfp* transgene, the location of restriction sites, and the sizes of digestion products of a non-methylated GFP transgene are shown in Figure 2(a). In non-silenced, infected tissue (Figure 2b, lane 1) only the two major *gfp*-specific fragments of 0.56 and 0.28 kb were detected. In contrast, in silenced, infected tissue additional fragments of 0.36, 0.84 and 1.3 kb were detected; the pattern of fragments was the same for all the samples (Figure 2b, lanes 2–4). This indicated a partial methylation at the three *Sau96*I sites internal to the *gfp* sequence, but no methylation at the flanking sites in the non-transcribed 35S and tnos portions of the transgenes. Incomplete RNA-directed methylation was also a feature of previous, related studies (Jones *et al.*, 1998, Jones *et al.*, 1999). The complete digestion of the DNA with *Sau96*I was confirmed by re-probing the Southern blot for *hsp70* DNA, as shown by the detection of just a single 1.4 kbp band in all lanes (Figure 2c).

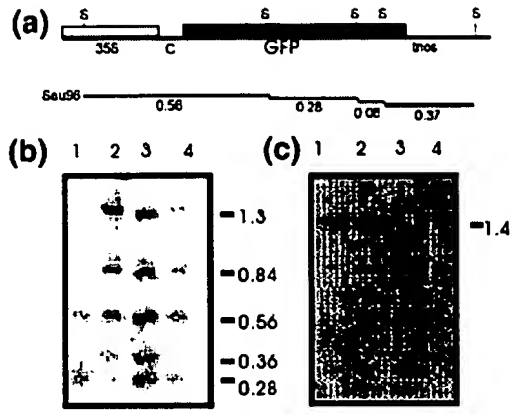

Figure 2. Methylation associated with PTGS of *gfp* initiated using short, homologous *gfp* sequences.
(a) Structure of the *gfp* transgene is shown as including the 35S promoter (35S; open box), the chitinase endoplasmic reticulum targeting signal sequence (C), *gfp* coding sequence (solid box), and the nopaline synthase terminator (tnos). *Sau96*I restriction sites and sizes of the expected digestion products in kilobases are shown below.
(b,c) Southern blot analyses of genomic DNA samples from tissue infected with PVX, non-silenced leaves (lane 1), PVX-GFP silenced (lane 2), PVX-GFP43 (43 nt *gfp*) silenced (lane 3), and PVX-GFP28 (28 nt *gfp*) silenced (lane 4) leaves of *gfp*-transgenic *N. benthamiana* plants. The same blot was probed sequentially with *gfp* (b) and *hsp70* (c) cDNAs. Sizes (in kilobases) of relevant DNA fragments are indicated.

Table 2. Silencing of *gfp* with PVX containing oligonucleotides (33 nt) with or without symmetrical cytosine residues

PVX-Oligo	Nucleotide position	Polarity (S/AS)	CNG or CG	Silencing (+/–)
A	786–818	S	No	+
B	786–818	AS	No	+
C	497–529	AS	No	+
D	508–540	S	Yes	+
E	508–540	AS	Yes	+
F	746–778	S	Yes	+
G	746–778	AS	Yes	+
GFP28	746–773	S	Yes	+
GFP	1–818	S	Yes	+
PVX	NA	NA	NA	–

Canonical CpG and CpNpGp are not essential for de novo *methylation for a GFP transgene*

The identification of short sequences capable of initiating PTGS allowed us to test the effect of specific RNA sequences for the capacity to induced methylation, particularly to address the importance of canonical CpG or CpNpG residues. Unfortunately, the shortest competent fragment for silencing (23 nt) did not allow *gfp*-specific sequences completely devoid of C residues to be tested. It has been suggested that methylation of symmetrically located Cs may provide nucleation centres for the spread of methylation to adjacent non-symmetrical C residues (Finnegan *et al.*, 1998). To test the significance of CpG or CpNpG for inducing methylation, two regions of *gfp* devoid of symmetrical C residues were identified, and corresponding S and AS synthetic oligonucleotides inserted into the PVX vector. The regions identified (Table 2) made it possible to use sequences of 33 nt, which had the advantage of increasing the efficiency of PTGS induction. Adjacent sequences containing symmetrical C residues were tested in parallel (Table 2). Unfortunately, the sense oligonucleotide corresponding to *gfp* nt 497–529 was unstable in the PVX vector, and could not be analysed further.

PVX-oligo-A to -G, PVX-GFP28, PVX-GFP and wild-type PVX were all agro-inoculated to *gfp-N. benthamiana* and scored for silencing after 25 days (Table 2). All the viruses carrying *gfp* sequences effectively initiated silencing. Although some constructs were more effective than others at 25 dpi, by 41 dpi the silencing from each construct was complete. This experiment was repeated five times using two plants per construct. The least efficient initiators of silencing were always the oligonucleotides devoid of symmetrical C residues, irrespective of orientation. For reference, these were always weaker than PVX-GFP28 (Figure 1b). No correlation between the strength of silencing and the number of symmetrical C residues in the initiator sequence could be made.

It was possible that the inefficient initiation of *gfp* PTGS by PVX-oligo-A to -C could be attributable to reduced transgene methylation as a result of triggering with a GFP fragment devoid of canonical cytosines. Hence genomic DNA isolated from fully PVX-oligo-silenced tissue at 22 dpi was digested with *Sau96*I and analysed by Southern blot hybridization with a GFP probe to assess the extent of methylation. In this case *Sau96*I digestion gave fragments of 0.56, 0.37 and 0.28 kb for non-silenced samples (Figure 3, lane 1), and additional fragments of 1.3 and 0.84 kb in silenced samples (Figure 3, lanes 2–9). Equivalent data were obtained using a second methylation-sensitive restriction enzyme, *Alu*I (data not shown). Hybridization of the same blots with a probe for *hsp70* confirmed that the pattern of fragments was not due to incomplete digestion of the DNA samples (data not shown).

PTGS targeted to gfp *using small oligonucleotides is able to target recombinant virus for degradation*

When PTGS is directed in a *gfp*-transgenic line by PVX-GFP, the strong silencing targets *gfp* mRNA and PVX-GFP RNA for degradation, and PVX-GFP is prevented from further accumulation (Ruiz *et al.*, 1998). This effect is a combination of the strength of the PTGS response and the potential of PVX containing all the *gfp* cDNA to be seen as a target for degradation. Experiments involving transgenic plants displaying constitutive PTGS-based virus resistance

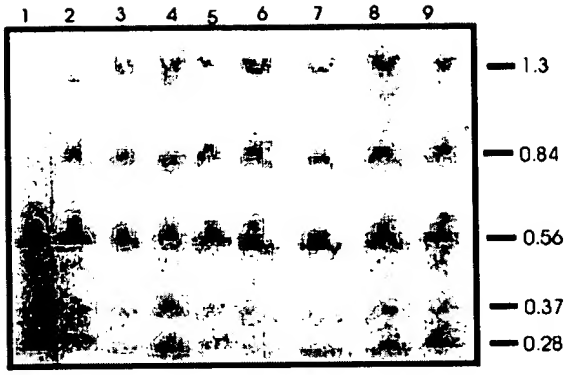

Figure 3. Methylation associated with PTGS of *gfp* induced with recombinant PVX carrying specific *gfp* oligonucleotides.
Southern blot analysis of genomic DNA samples from non-silenced tissues infected with PVX (no insert) (lane 1), and silenced tissues infected with PVX-GFP28 (28 nt; lane 2) or PVX-GFPoligo-A to -G (lane 3–9). The blot was probed with *gfp* cDNA. Sizes (in kilobases) of relevant DNA fragments are indicated.

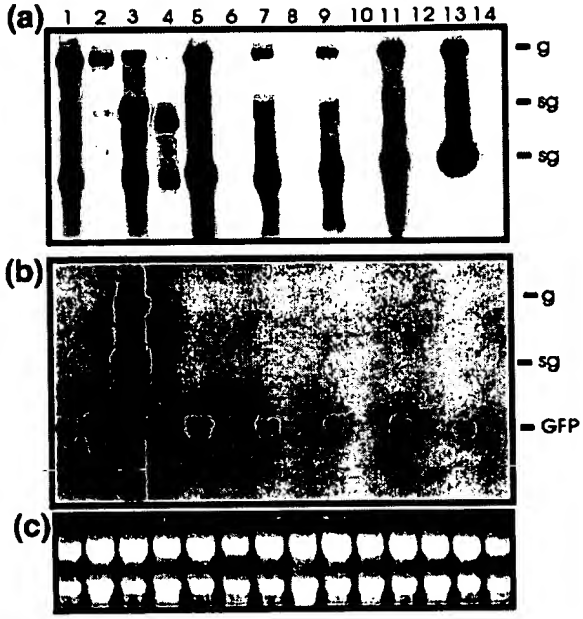

Figure 4. PTGS-mediated targeting of PVX RNA and *gfp* mRNA species.
Northern blot analysis of samples from non-silenced (lanes 1, 2, 3, 5, 7, 9, 11, 13) or silenced (lanes 4, 6, 8, 10, 12, 14) tissues infected with non-recombinant PVX (lanes 1 and 2), PVX-GFP (lanes 3 and 4), PVX-GFP28 (lanes 5 and 6), PVX-oligo-A (S and lacking symmetrical C residues; lanes 7 and 8), PVX-oligo-B (AS and lacking symmetrical C residues; lanes 9 and 10), PVX-oligo-D (S and with symmetrical C residues; lanes 11 and 12) or PVX-oligo-E (AS and with symmetrical C residues; lanes 13 and 14). Non-silenced tissues were harvested at 14 dpi, except for the sample in lane 2, which was harvested with silenced tissues at 27 dpi. Total RNA (10 μg) was probed for either (a) PVX-specific or (b) GFP-specific sequences. Equal gel loadings were confirmed by ethidium bromide staining of ribosomal RNAs (c). The positions of the genomic (g) and subgenomic (sg) PVX RNAs and the *gfp* transgene mRNA (GFP) are marked.

have identified 60 nt as being the smallest region of homology able to tag a recombinant virus for degradation (Sijen *et al.*, 1996). To determine if regions of homology of less than 60 nt were able to identify the recombinant PVX RNAs as targets in a *de novo*-directed PTGS system, and whether the canonical C content might influence the efficiency of targeting, GFP and PVX RNA levels were assessed in tissues silenced by PVX-GFP, PVX-GFP28 and PVX-oligo-A, -B, -D and -E (Table 2). Total RNA from infected, non-silenced tissue 14 dpi, and from silenced tissue 27 dpi, was subjected to Northern analysis using probes for PVX (Figure 4a) or *gfp* (Figure 4b) sequences. The PVX probe detected genomic and subgenomic RNAs for both PVX-GFP and PVX. The *gfp* probe detected *gfp*-transgene mRNA and PVX-GFP; PVX-GFP28 or PVX-oligo RNAs were not detected. The levels of PVX RNA, that accumulated in leaves of *gfp*-transgenic plants at 14 dpi, and in upper leaves at 27 dpi, are shown (Figure 4a, lanes 1 and 2). As previously demonstrated for PVX-GFP (Ruiz *et al.*, 1998), the levels of viral RNAs in the silenced tissue at 27 dpi (Figure 4a, lanes 4, 6, 8, 10, 12, 14) were dramatically reduced compared to the non-silenced tissue at 14 dpi (Figure 4a, lanes 3, 5, 7, 9, 11, 13). The mobility shift of the subgenomic PVX RNA in Figure 4(a), lane 4, and the absence of hybridization with the *gfp* probe (Figure 4b, lane 4), indicates that residual PVX RNA in silenced tissue results from recombination. The absence of recombined PVX in tissues silenced using PVX-oligo suggests that the smaller inserted sequences reduce the propensity for recombination (Figure 4, lanes 5–14). This was confirmed by RT–PCR analysis of extracts of infected plants using primers that enabled the detection of recombinant and wild-type PVX (data not shown). When the 14 and 27 dpi RNA samples were analysed using the *gfp* probe, the expected dramatic reduction in *gfp* mRNA levels was observed in silenced tissues (27 dpi; Figure 4b, lanes 4, 6, 8, 10, 12 and 14). Actually, *gfp* mRNA was reduced even at 14 dpi in tissues infected with PVX-GFP (Figure 4b, lane 3), indicating that the mRNA was more prone to degradation than the virus at this time. The equal loss of PVX-oligo-A, -B, -D and -E RNAs at 27 dpi showed that the content of symmetrical cytosine residues had no influence on the mechanism of RNA targeting and degradation. The loss of these RNAs and PVX-GFP28 also showed that homology as short as 28 nt was sufficient to provide an effective target.

PTGS of an endogenous gene using short regions of homology

To determine whether PTGS of an endogenous gene could be initiated by short regions of homology in PVX, oligonucleotides were designed to different regions of the endogenous phytoene desaturase gene (*pds*). This is a

Table 3. Silencing of phytoene desaturase with PVX containing PDS-specific oligonucleotides

PVX-PDSoligo	Size (nt)	Nucleotide position	Polarity (S/AS)	Silencing (+/–)
1	34	1326–1359	S	–
2	34	1326–1359	AS	–
3	52	1326–1381	S	–
4	52	1326–1381	AS	+
5	33	1498–1530	S	+
6	33	1498–1530	AS	+
7	51	1498–1548	S	+
8	51	1498–1548	AS	+
9	34	1639–1672	S	–
10	34	1639–1672	AS	–
PDS	368	1322–1690	S	+
PVX	NA	NA	NA	–

single-copy, low expressed gene in *N. benthamiana* which has been shown to be susceptible to virus-induced PTGS with a 368 bp fragment of the *N. benthamiana pds* gene (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998). Silencing of *pds* causes suppression of carotenoid biosynthesis so that the affected plants become susceptible to photo-bleaching (Demmig-Adams and Adams, 1992).

Recombinant PVX were constructed carrying *pds* S and AS oligonucleotides specific to different regions within the 3′ half of *N. benthamiana pds* (Table 3). The oligonucleotides, including the contribution from flanking nucleotides in the PVX cloning site, were either 33, 34, 51 or 52 nt, sizes that reproducibly gave strong silencing of the *gfp* transgene. The *pds* sequences were cloned in both orientations into the PVX vector and agro-inoculated onto *N. benthamiana*. As a positive control, 368 bp of *pds* from *N. benthamiana* (corresponding to 1322–1690 nt of the tomato cDNA (Kumagai *et al.*, 1995; Pecker *et al.*, 1992) was used. At 25 dpi plants were scored for the presence of photo-bleaching, indicative of silencing of PDS (Table 3). Unlike the situation with PVX-stimulated silencing of the *gfp*-transgene with sequences longer than 23 nt, not all the PVX-PDSoligo constructs were able to initiate silencing. Broadly, they fell into two classes: those that did, and those that did not cause photo-bleaching (Table 3). Sequences from the *pds* region including nts 1498–1548 were effective irrespective of orientation, whereas the flanking regions were generally ineffective, the exception being AS oligonucleotide 4 (nts 1326–1381). Hence plants infected with PVX-PDSoligos –4 to –8 (Figure 5a, panels 4–8) all showed photo-bleaching, albeit to different degrees. Plants infected with PVX-PDSoligos1–3, 9 and 10 (Figure 5a, panels 1–3, 9, 10) failed to show photo-bleaching, even after 45 d.p.i.

The wide variation in phenotype (more-or-less photo-bleaching) amongst those sequences effective for *pds* silencing revealed some trends. Photo-bleaching was strongest when triggered by sequences in the AS rather than the S orientation (Figure 5a, compare panels 5 and 6; 7 and 8, where 6 and 8 result from the action of AS *pds* sequences 6 and 8). This orientation bias was not observed with larger fragments of *pds* (Ruiz *et al.*, 1998). To confirm that the phenotype related to *pds* mRNA levels, RNA samples from photo-bleached leaves were subjected to semiquantitative duplex RT–PCR (Figure 5b). In comparison with the relative accumulation of *pds* and *ubiquitin* mRNAs in non-silenced tissues infected with PVX (without PDS sequences), both PVX-PDSoligo-7 (S) and PVX-PDSoligo-8 (AS) infections led to a reduction (relative to *ubiquitin*) of *pds* mRNA. This was marginal for PVX-PDSoligo-7, but clear for PVX-PDSoligo-8. It also appeared that larger oligonucleotides (51–52 nt) were more efficient than smaller oligonucleotides (33–34 nt) at initiating silencing (Figure 5a, compare panels 2 and 4; 5 and 7; 6 and 8). However, in the case of PVX-PDSoligo-5 to –8 (Figure 5a, panels 5–8), which cover the same area of *pds*, the AS oligo-6 (33 nt; Figure 5a, panel 6) was more efficient than the S oligo-7 (51 nt; Figure 5a, panel 7). This indicates that orientation may have a stronger influence than size on the silencing of *pds*.

We have previously demonstrated that *de novo* methylation is not associated with silencing of the endogenous gene *rbcs* (Jones *et al.*, 1999). To determine if the same was true of silencing triggered by PVX-PDS or a PVX-PDSoligo, DNA was isolated from *pds* silenced leaf tissue and analysed using methylation sensitive enzymes, *Hin*dIII, and *Hae*III. When probed with *pds* cDNA, there was an identical hybridization profile obtained for both non-silenced and silenced leaf tissue, indicating that *de novo* methylation is not associated with silencing of *pds* (data not shown).

Discussion

By using random fragments of *gfp* and *gfp* oligonucleotides in a virus vector, we were able to assess indirectly the influence of size and sequence on the capacity to direct PTGS *de novo* to *gfp* mRNA in *gfp*-transgenic plants. The shortest length of *gfp* homology with the ability to target silencing to *gfp* mRNA was 23 nt. This correlates well with the size of small RNAs associated previously with PTGS in plants (Hamilton and Baulcombe, 1999; although originally sized at ≈25 nt, improved techniques have provided a more accurate size assessment as 21 and 23 nt, unpublished data), and with the size requirement for RNAi in other systems. Fortuitously, the failure to observe silencing with the 27 nt with incomplete identity with *gfp* (due to the presence of a sequence error) showed that it was necessary for the short initiating sequence to have complete homology with the target. One likely consequence is

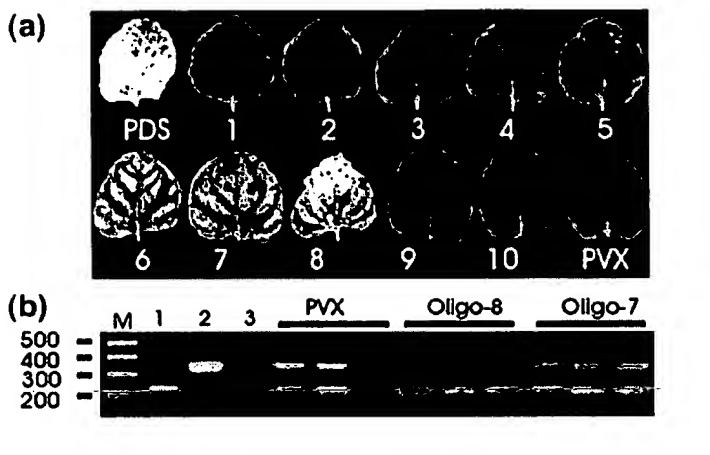

Figure 5. Correlation of the *pds*-silenced phenotype with *pds* mRNA levels.
(a) Phenotype PVX-PDSoligo-1 to -10 infection on the upper leaves of *N. benthamiana* at 26 dpi in relation to that seen after infection with PVX-PDS or PVX (without insert).
(b) Ethidium bromide-stained gel of products obtained after RT–PCR analysis of mRNAs from tissues infected with PVX, PVX-PDSoligo-7 or PVX-PDSoligo-8. Three samples from each of three individual plants were analysed using primers for *ubiquitin* mRNA (lower band) and *pds*; a representative from each plant is shown. Control reactions with RNA isolated from PVX-infected tissue (non-silenced) were carried out separately using primers for *ubiquitin* (lane 1) and *pds* (lane 2). The corresponding bands were never seen in the absence of reverse transcriptase (lane 3). The sizes (bp) of the markers (M) are indicated on the left of the gel.

that the occurrence of PTGS will be determined by the presence or absence of stretches of 23 nt of identity rather than by the mean (percentage) homology between inducer and target.

The efficiency of initiation of silencing increased dramatically when the size of the *gfp* fragment was increased by only a few nt. This may reflect the increase in probability that the exact 23 nt of *gfp* would be generated by a processive cleavage of ds PVX-GFPFrag RNA, as proposed for the cleavage mechanism in *Drosophila* cell-free extracts. The lower size limit for PTGS initiation at 23 nt not only provides evidence that they have the potential to act as signals for inducing PTGS, but also provides an experimental link between the physical identification of small RNAs in plants and their function in *Drosophila* cell-free extracts.

The quantitative nature of the silencing response with short homologous sequences has also been noted for RNAi in *Trypanosoma brucei* (Ngô *et al.*, 1998) and in *Drosophila* cell-free extracts (Tuschl *et al.*, 1999). In the former, 59 nt of homology induced mRNA degradation, but the effect was much stronger with 100–450 nt. In the cell-free extracts, weak RNA degrading activity was directed by dsRNA of 149 nt of homology, but 505 nt was markedly stronger. Surprisingly, a 49 nt RNA was inactive (Tuschl *et al.*, 1999), although the 21–23 nt fraction purified following cell-free RNAi was active in targeting RNA in a new reaction (unpublished data in Zamore *et al.*, 2000).

Logically, if short homologous regions are capable of inducing PTGS, we might expect them to be effective in targeting homologous RNA in the cytoplasm. Previously, Sijen *et al.* (1996) showed that as little as 60 nt homology between a recombinant PVX vector and a transgene could target the virus for degradation to give resistance. Our data show that the same effect can be achieved with just 28 nt homology. In contrast, when silenced transgenes composed of fragments of the tomato spotted wilt virus (TSWV) N gene fused to *gfp* were analysed for their ability to target TSWV, resistance was seen only when >110 nt of N were present in the transgene (Pang *et al.*, 1997). In our experiments, the source of the 28 nt homology would be the sum of the RNA degradation products from the recombinant virus and the transgene mRNA, conceivably a higher dose than found in the other experimental system.

We attempted to use short, homologous RNA sequences to silence an endogenous gene (PDS). While this was effective in some cases, particularly for sequences in the centre of the region analysed, the effect was not reproducible even when 51–52 nt fragments were used. As for the shorter *gfp* homologous sequences, effectiveness was also variably influenced by sequence orientation. The reason for this is unknown when the likely source of the PTGS inducer is viral dsRNA. However, analysis of the silenced plants reinforces the view that there is a fundamental difference between endogenous genes and transgenes in the interaction of cytoplasmically derived RNA and genomic DNA, reflected in their methylation status in silenced tissues. There was no *de novo* methylation of *pds*.

As silencing directed by recombinant RNA viruses probably has the capacity to trigger the degradation of existing homologous mRNAs in the cytoplasm, we could not determine with certainty whether the short sequences we tested could interact directly with genomic DNA, potentially to direct *de novo* methylation. However, the influence of symmetrical C residues on the efficiency of PTGS might indicate a direct interaction from the input recombinant virus. Also, it is clear from the RNA-directed methylation of transgenic viroid sequences (Pélissier and Wassenegger, 2000) that short homologous DNA

sequences (30 bp) can be invoked as targets for methylation. Why we found that PVX-GFP28 (28 nt *gfp*) led to DNA methylation, but PVX-PDSoligo-6 (33 nt *pds*) did not, even though both infections initiated silencing, remains to be determined. However, it would appear that the plant can distinguish between a transgene and an endogenous gene as substrates for RNA-directed methylation. Surprisingly, PTGS induced by ds viral RNA carrying a very short homologous region led to methylation throughout the transcribed region of the transgene. Since the transgene mRNA appeared to be more susceptible than the viral RNA to targeted degradation (Figure 4b), it is conceivable that it could act as the primary target in the cytoplasm of the short region of sequence homology from the virus. The processive degradation of the target mRNA (Zamore *et al.*, 2000) could release further *gfp* fragments that additively direct methylation throughout the transcribed region of the transgene. This can also lead to subsequent targeting of RNAs with homology to adjacent regions (Jones *et al.*, 1999; Ruiz *et al.*, 1998). Whether methylation is just an indicator of this capacity for spreading the PTGS specificity, or whether it is an active component, remains a key question.

Experimental procedures

Plant material

Transgenic *Nicotiana benthamiana* plants (line 16c) carrying a single 35S::*gfp*::tnos transgene have been described previously (Ruiz *et al.*, 1998).

Recombinant PVX viruses

Fragments of GFP5 (Haseloff *et al.*, 1997) DNA were generated by limited DNaseI digestion in the presence of Mn^{2+} (Melgar and Goldthwait, 1968). The digested DNA was size-fractionated in a 1.5% agarose gel and fragments of <100 bp cloned into the *Sma*I site of a PVX vector (pGR107; Jones *et al.*, 1999), adjacent to a duplicated subgenomic coat protein promoter. Cloning synthetic oligonucleotides into the *Sma*I site similarly generated other recombinant PVXs. In determining the precise size of the gfp-homologous sequence in the PVX vector, the contribution of the sequences comprising the *Sma*I cloning site were also taken into account. The vector pGR107 expresses an infectious PVX RNA from a CaMV 35S promoter after introduction into plant cells using *Agrobacterium tumefaciens* pGV3101 stab inoculation ('agro-inoculation'). PVX-GFP contained a full-length gfp cDNA cloned into the *Sma*I site (Ruiz *et al.*, 1998). PVX-PDS similarly contained a 368 bp fragment of *N. benthamiana* pds cDNA (Ruiz *et al.*, 1998). Varying numbers of plants (three to ten) were agro-inoculated with the PVX constructs discussed in the text. Without exception, all plants infected with the same construct gave a consistent phenotype (either they did or did not initiate silencing).

GFP imaging

Observation and photographic recording of GFP fluorescence was as previously described (Voinnet *et al.*, 1998).

Southern blot analysis

Genomic DNA was extracted from leaves using the 'DNAeasy' kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. DNA digestion with methylation-sensitive restriction enzymes and gel-blot analysis was as described (Jones *et al.*, 1998). ^{32}P-labelled hybridization probes corresponded to the entire gfp sequence, a 368 bp *N. benthamiana* pds cDNA fragment or 450 bp of the *N. benthamiana* heat-shock protein 70 (hsp70) cDNA.

RNA extraction and analysis

Total RNA was extracted using RNA isolator (Genosys Biotechnologies Inc., The Woodlands, TX, USA) following the manufacturer's instructions. RNA electrophoresis and gel-blot analysis were performed as described previously (Jones *et al.*, 1998) and hybridized with *gfp and* PVX probes. For semiquantitative RT–PCR analysis, three leaves showing the *pds* silenced phenotype were sampled from each of three individual plants infected with either PVX-PDSOligo-7 or -8, or from similarly aged leaves infected with PVX. Poly(A)+ RNA was isolated from 10 µg total RNA using Dynabeads (Dynal AS, Oslo, Norway) as per the manufacturers instructions. cDNA was synthesized using Expand reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany), and used in a duplex PCR reaction containing oligonucleotides specific for amplifying *ubiquitin* and *pds* mRNAs. Semi-quantitative PCR of cDNA derived from the equivalent of 1 µg of total RNA was performed using the following conditions: 95°C/5 min for 1 cycle, 95°C/30 sec, 55°C/1 min, 72°C/1 min for 22, 26 or 30 cycles and 72°C/10 min for 1 cycle, for each sample. The linear phase of DNA amplification (26 cycles) was determined by electrophoresing the PCR products on a 1.5% agarose gel. The *pds* oligonucleotides were designed to detect *pds* mRNA and not the *pds* sequences within PVX-PDSOligo-7 or -8.

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